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Control of Antigen Presentation by a Single Protease Cleavage Site

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Summary

Protein antigens require limited proteolytic processing to generate peptides for binding to class II MHC molecules, but the proteases and processing sites involved are largely unknown. Here we analyze the effect of eliminating the three major asparagine endopeptidase (AEP)-processing sites in the microbial antigen tetanus toxin C fragment. The mutant antigen is highly resistant to proteolysis by AEP and crude lysosomal extracts and is dramatically impaired in its ability to be processed and presented to T cells. Remarkably, processing at a single asparagine residue (1219) is obligatory for optimal presentation of many T cell epitopes in this antigen. These studies demonstrate that cleavage at a single processing site can be crucial for effective antigen presentation.

Introduction

Proteases perform two key events in the class II MHC antigen processing pathway. First, they degrade endocytosed protein antigens to liberate peptides for binding to class II MHC molecules (Wolf and Ploegh, 1995; Fineschi and Miller, 1997; Watts, 1997). Second, they initiate the removal of the invariant chain (Ii) chaperone, which targets newly synthesized class II MHC molecules to the endocytic pathway (Blum and Cresswell, 1988; Chapman, 1998). Until recently little was known about the proteases required for either of these events. Surprisingly, mice lacking the major cathepsins B and D had little obvious defect on either maturation of class II MHC molecules or on the presentation of protein antigens to T cells (Deussing et al., 1998). These results raised the possibility that the protease complement of the endosome/lysosome system is redundant and that any one of several enzymes can perform the proteolytic events needed for effective antigen presentation. However, mice lacking cathepsin S or cathepsin L clearly have specific defects in class II MHC maturation. Cathepsin S has emerged as a key enzyme in the later stages of (Ii) processing in most cell types. Chemical inhibition of this enzyme (Riese et al., 1996) or its deletion in cathepsin S null mice (Nakagawa et al., 1999; Shi et al., 1999) leads to an accumulation of class II MHC molecules with partially processed Ii fragments in most class II MHC-positive cell types, including dendritic cells (Driessen et al., 1999). Germinal center formation and Ig class switching were defective in the null mice (Shi et al., 1999) as was the autoimmune response to collagen in DBA1/LacJ mice (Nakagawa et al., 1999). In contrast, cathepsin L null mice showed normal class II MHC maturation in splenic cells but incomplete degradation of the Ii in cortical thymic epithelial cells (which do not express cathepsin S) and as a result, altered positive selection of $CD4^+$ T cells (Nakagawa et al., 1998). Thus, the development of specific inhibitors and the generation of protease gene-targeted mice has begun to allow some of the key proteases in Ii processing to be identified.

In contrast, very little information exists concerning the proteases involved in antigen processing and the sites that they cleave in protein antigens. As noted above, chemical inhibition of specific proteases or protease gene knockouts may affect Ii processing as well as antigen processing, potentially complicating the dissection of the role of proteases in the overall process. An alternative approach has been to digest native or denatured protein antigens in vitro with defined proteases to assess whether T cell epitopes are liberated or not. Cathepsins D, E, and B have all been shown to be capable of liberating epitopes from diverse protein antigens (Rodriguez and Diment, 1992; van Noort and Jacobs, 1994; Hewitt et al., 1997), but this type of analysis cannot establish that the enzyme in question actually does the job in living cells. Different scenarios can be envisaged regarding protease requirements for antigen processing. One possibility is that multiple enzymes and multiple alternative processing sites can serve to liberate T cell epitopes. On the other hand, a more defined processing program may be required for optimal epitope presentation (Bogyo and Ploegh, 1998). No information exists to date on this point. Recently, we identified a novel cysteine protease activity in the class II MHC pathway (Manoury et al., 1998). Unlike other lysosomal proteases, which have rather broad specificity (Chapman, 1998), this enzyme cleaves only after asparagine residues (Chen et al., 1997; Dando et al., 1999). This asparaginyl endopeptidase (AEP), which is homologous to the legumain or hemoglobinase family of cysteine proteases, emerged as the dominant proteolytic activity when lysosomal extracts were used to process the microbial antigen tetanus toxin C fragment (TTCF) in vitro. Suppression of the activity of this enzyme in human B cells inhibited presentation of this antigen, while preprocessing of TTCF with AEP accelerated presentation (Manoury et al., 1998).

These results allowed us to propose for the first time a pathway for processing of a foreign antigen in which cleavage by AEP initiates and is rate limiting for efficient presentation to T cells. Further refinement of this model is however both possible and desirable. First, inhibition of AEP activity did not reveal which processing sites in TTCF were responsible for efficient T cell epitope presentation. Second, as noted above, protease blockade either by chemical inhibition or by gene knockout

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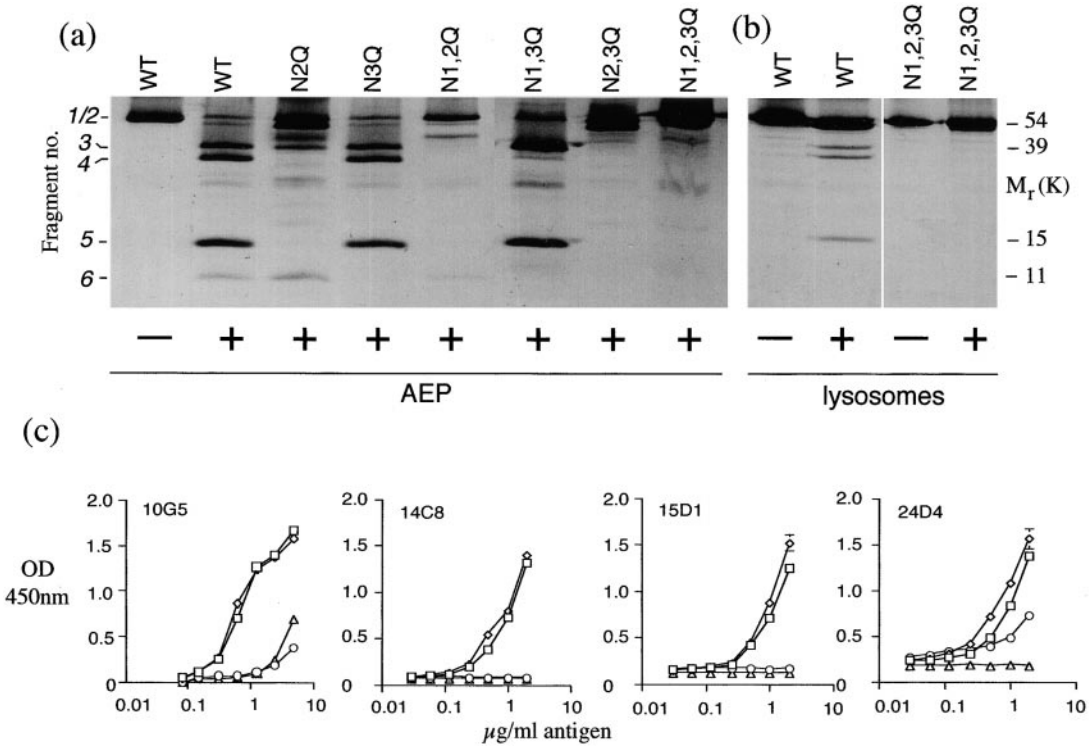

Figure 1. Mutation of AEP-Processing Sites Eliminates Digestion by AEP and by Purified Lysosomes but Does Not Alter Tertiary Conformation

Wild-type TTCF and N/Q mutants were digested either with AEP (a) or purified B cell lysosome fractions (b) under conditions described in Experimental Procedures; the products were analyzed on a 15% Tris-tricine SDS gel. Digestion of wild-type TTCF produces five Coomassie blue–stained products (numbered left of [a]). Specific mutations eliminate specific products. Processing products of the N1Q mutant lack bands 2 and 4 (data not shown). All products are missing when the N1,2,3Q mutant was used as substrate. ELISAs (c) were performed to test the conformational integrity of different TTCF mutants. TTCF (diamonds), N1,2,3Q mutant (squares), and two other TTCF mutants, YM $^{1148/9}$ to FK $^{1148/9}$ (triangles) and YY$^{1165/6}$ to FK$^{1165/6}$(circles), were titrated across 96-well EIA plates and incubated with four culture supernatants (10G5, 14C8, 15D1, and 24D4) selected from a panel of conformationally specific antibodies. Antibody binding was detected using an HRP-conjugated goat anti-mouse IgG and TMB substrate. OD_{450nm} absorbance readings are given as means of triplicate points. The N1,2,3Q mutant was indistinguishable from wild type in this assay.

might affect additional events besides antigen processing, including Ii processing and potentially processing of pro-protein precursors. Ideally, a rigorous test of the requirement of a given protease for T cell epitope generation in a specific antigen should not eliminate the protease but rather the processing sites in the antigen. To address these issues and to refine our model, we have used site-directed mutagenesis to systematically eliminate AEP-processing sites in the TTCF antigen. Our results demonstrate that elimination of specific processing sites in the TTCF antigen can dramatically affect the efficiency of antigen presentation and unexpectedly reveal that cleavage at a single site is crucially important for presentation of a wide range of T cell epitopes in this microbial antigen.

Results

Mutation of Specific Asparagine Residues Eliminates AEP Processing In Vitro

Our previous studies showed that the C fragment domain of the TTCF antigen is cleaved by AEP after asparagine residues 873, 1184, and 1219 (Manoury et al., 1998). The specificity of AEP suggested that mutation of these residues to glutamine would prevent processing at these sites. A strict test of the importance of this protease in TTCF processing should then be possible under conditions where the enzyme is fully active. Several forms of TTCF were generated in which one, two, or all three of the above asparagine residues were mutated to glutamine. For convenience, the three mutated sites are referred to as N1Q, N2Q, and N3Q, respectively.

We first assessed the processing of these mutants by both purified AEP and lysosomal fractions. As shown in Figure 1a, mutation of the major AEP-processing sites resulted in an altered processing pattern fully consistent with our earlier mapping of these sites (Manoury et al., 1998). For example, mutation of site 2 (N2Q) prevents the generation of fragment 5, while mutation of site 3 (N3Q) eliminates fragment 6. Loss of two sites resulted in more drastic inhibition of processing (e.g., N1,2Q), and mutation of all three major AEP sites produced a form of TTCF that is highly resistant to AEP processing (Figure 1a). We also tested the capacity of the N1,2,3Q mutant to be processed by unfractionated lysosomal hydrolases. Highly purifed lysosomes were prepared from the murine B cell IIA1.6 by a combination of sucrose density gradient centrifugation and free-flow electrophoresis (Amigorena et al., 1994). In the presence of this

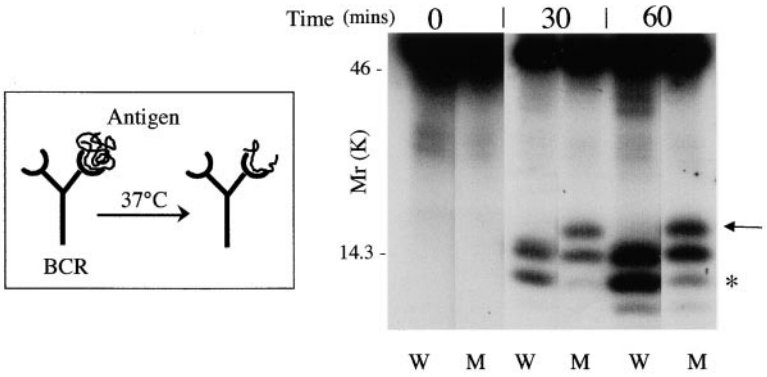

Figure 2. Processing of the N1,2,3Q Mutant Is Altered Compared to TTCF

Wild-type (W) and N1,2,3Q mutant (M) antigens were iodinated, allowed to bind for 1 hr on ice to LB27.4 murine B cells transfected with the human 11.3 BCR (see Experimental), and after washing to remove unbound antigen, incubated at 37°C for times as shown. BCR-associated processing products were analyzed on 15% Tris-tricine gels as described in Experimental Procedures. The 11.3 BCR stabilizes or "footprints" a distinct set of processing products following TTCF processing (Davidson and Watts, 1989; see explanatory inset left of figure). Arrow indicates a unique product following N1,2,3Q processing. Asterisk indicates a lost or more slowly produced product.

mixture of enzymes, wild-type TTCF was processed to give an identical pattern to that seen previously with purified human B cell lysosomes, while the N1,2,3Q mutant was highly resistant to lysosomal proteolysis (Figure 1b). This confirms that AEP initiates lysosomal TTCF processing in vitro and that processing of this antigen can be manipulated by the relatively subtle change of an asparagine residue to glutamine. We confirmed that the tertiary conformation of TTCF was not detectably affected by this mutagenesis using a panel of conformation-specific monoclonal antibodies (A. N. A. et al., unpublished data). Binding of four members of this panel to wild-type TTCF, N1,2,3Q TTCF, and two unrelated mutant TTCFs (YM $^{1148/9}$ to FK$^{1148/9}$ and YY$^{1165/6}$ to FK$^{1165/6}$), which are known to be conformationally altered (data not shown), were tested following coating onto microtiter plates. TTCF and N1,2,3Q TTCF were equally capable of binding to these and other conformation-sensitive monoclonal anti-TTCF antibodies, whereas the YM $^{1148/9}$ to FK$^{1148/9}$ mutant bound poorly to most of these antibodies (Figure 1c). The YY$^{1165/6}$ to FK$^{1165/6}$ mutant was able to bind to some members of the panel but not all (Figure 1c).

Altered In Vivo Processing of Mutated Antigens

We next sought evidence that loss of AEP-processing sites would affect TTCF processing in living cells. Antigen proteolysis has been difficult to follow in living cells because the proteolytic products are short lived unless captured by class II MHC molecules (Donermeyer and Allen, 1989; Davidson et al., 1991; Castellino et al., 1998). However, processing of antigen/antibody complexes can yield fragments that are long lived due to a protective or "footprinting" effect of antibody (Davidson and Watts, 1989; Simitsek et al., 1995). To assess what impact, if any, loss of AEP-processing sites would have on antigen proteolysis in vivo, we generated murine B cells (LB27.4) transfected with the heavy and light chains of the BCR from the human anti-tetanus EBV line 11.3 (Lanzavecchia, 1985; Knight et al., 1997). Mutant and wild-type antigens were iodinated, bound to surface BCR at 4°C, and their fate followed by shifting the cells to 37°C. At each time point, proteolytic fragmentation of antigen was analyzed by SDS-PAGE of either whole cell lysates or following recovery of 11.3 BCR-associated antigen fragments (see Experimental Procedures). As shown in Figure 2, 11.3 BCR-mediated uptake of wild-type iodinated TTCF produced a pattern of labeled 11.3 BCR-associated TTCF fragments similar to those seen before in human 11.3 B cells (Davidson and Watts, 1989). An essentially similar pattern was seen in whole cell lysates (data not shown), confirming that at this level of resolution, only BCR-associated fragments persist following antigen proteolysis. In contrast, while processing of the mutant N1,2,3Q TTCF clearly occurred, albeit more slowly (e.g., band labeled with an asterisk in Figure 2); in living cells, the processing pattern was quite distinct. In particular, a new larger footprinted antigen fragment was clearly generated (Figure 2, arrow). This result provides direct biochemical evidence that loss of AEP cleavage sites affects antigen proteolysis.

Elimination of AEP-Processing Sites Blocks Presentation of T Cell Epitopes

We next assessed the presentation of T cell epitopes from TTCF lacking major AEP-processing sites using a newly generated panel of H-2^b and H-2^d-restriced TTCF-specific murine T cell clones and hybridomas recognizing several different regions of TTCF (A. N. A. et al., unpublished data; see Experimental Procedures for epitope sequences). To avoid BCR-mediated modulation of TTCF presentation (Figure 2; Simitsek et al., 1995), we elected to use the nonspecific B cell LB27.4 (H-2^{bxd}) as an antigen-presenting cell. We first analyzed the kinetics of presentation of T cell epitopes in TTCF and the N1,2,3Q mutant. Cells were pulsed with wild-type and mutant TTCF for different times, fixed lightly in aldehyde, and cocultured with different T cell hybridomas and clones. Presentation of most T cell epitopes in wild-type TTCF was detectable after 30 min of antigen pulsing, while presentation of the same T cell epitopes in TTCF lacking all three major AEP-processing sites was barely detectable even after 4 hr of antigen pulsing for some clones (Figure 3a). Both an H-2^b-restricted T cell clone (10A2) and an H-2^d-restricted hybridoma (2B1) were profoundly affected. Other H-2^b (4E10) and H-2^d (3A5) restricted hybridomas were also affected, albeit to a somewhat lesser extent (Figure 3a). These results are consistent with the idea that processing of TTCF by AEP is rate limiting for presentation of most T cell epitopes in TTCF (Manoury et al., 1998). It should be emphasized that none of these mutations actually fell

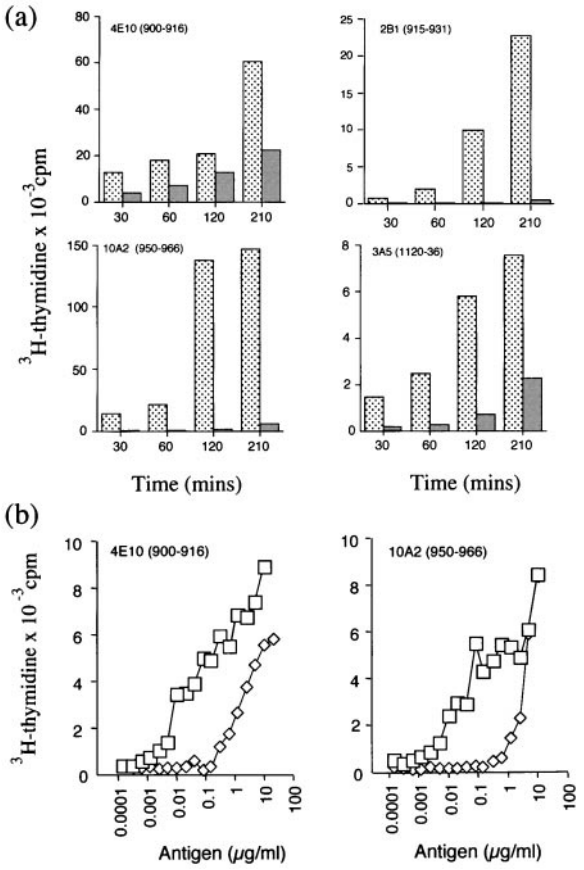

Figure 3. Loss of AEP-Processing Sites Leads to Inefficient Antigen Presentation

(a) TTCF (stippled bars) or the N1,2,3Q mutant (gray bars) were incubated with LB27.4 cells (5×10^6 cells/ml; 300 μg/ml antigen) and chased at 37°C for 30, 60, 120, and 210 min. The cells were then washed, fixed in 0.5% PFA, and cocultured for 24 hr with T cell hybridomas 4E10, 2B1, and 3A5 or T cell clone 10A2. Supernatants were tested for IL-2 production by assaying proliferation of the IL-2 dependent HT-2 cell line. Presentation at time zero was background.

(b) Diminished T cell presentation is evident with splenic APCs in dose-response titrations. TTCF (squares) or N1,2,3Q mutant (diamonds) were titrated as above with 1×10^5 splenic APCs/well and either 1×10^5 4E10 cells or 1×10^4 10A2 cells. IL-2 production was assayed as in (a).

within the T cell epitopes themselves. The N1,2,3Q mutant was also tested in dose-response titrations using either the murine B cell LB27.4 (data not shown) or splenic B cells from C57BL/6 mice (Figure 3b). Most T cell clones and hybridomas tested required 100- to 1000-fold more mutant TTCF to induce the same extent of IL-2 release compared to wild-type TTCF (Figure 3b). Taken together, these results provide direct evidence that elimination of processing sites in a protein antigen influences T cell epitope presentation.

Processing after FcR-Mediated Antigen Uptake Also Requires AEP-Processing Sites

Since the above assays depended on endocytosis of TTCF by nonspecific means, we also tested both wild-type and mutant TTCFs in assays where antigen uptake was receptor mediated. To do this we utilized murine IIA1.6 B cells transfected with the B2 isoform of FcγRII (Amigorena et al., 1992). FcR-mediated antigen capture was driven by inclusion of the anti-TTCF murine monoclonal antibody 10G5, recently generated in our laboratory (A. N. A. et al., unpublished data; Figure 1c). Presentation of the wild-type TTCF was detectable in the absence of the 10G5 antibody in the IIA1.6/FcγRIIB2 cells but was considerably augmented by its presence (Figure 4). In contrast, inclusion of the same antibody in the presence of the N1,2,3Q mutant gave no presentation to either of the T cell hybridomas tested (3A5 and 4A8) under the same conditions (Figure 4). Since both wild-type and mutant TTCF bind equally well to the 10G5 antibody (Figure 1c), we conclude that in B cells processing by AEP is necessary for antigen taken up by both specific (FcR) as well as nonspecific means.

A Single AEP-Processing Site Is Crucial for Efficient Antigen Presentation

The requirement of AEP for processing of TTCF might take several different forms. One possibility is that cleavage at all three major sites is necessary. Alternatively, cleavage at any one of the three sites may suffice to initiate the processing pathway. A third scenario is that cleavage at one of the three sites might have a dominant effect on subsequent events. To distinguish between these possibilities, we tested single-site mutants in T cell assays using LB27.4 as antigen-presenting cells. Loss of the first site, which is close to the N terminus and removes 25 residues, most of which correspond to the histidine tag, had no effect on the presentation of TTCF (data not shown). Individual mutation of the other two processing sites, however, produced a striking result. While loss of Asn 1184 (N2Q) had little impact on presentation of TTCF to T cells, mutation of Asn 1219 (N3Q) to glutamine had a deleterious effect that was essentially indistinguishable from the triple mutant in both dose-response titrations and in kinetic assays (Figures 5a and 5b). Presentation of TTCF lacking Asn 1219 lagged up to 4 hr behind wild-type TTCF in kinetic assays (Figure 5b) and is substantially worse in dose-response titrations (Figure 5a). Once again, the majority of T cell epitopes were affected by the loss of the Asn 1219 AEP-processing site. (Note that the epitope recognized by clone A8 [1225–1241] is sensitive to aldehyde fixation and therefore could not be tested in the kinetic assay). Mixtures of TTCF and the N3Q mutant were presented identically to TTCF alone, eliminating the unlikely possibility that the N3Q protein nonspecifically inhibited events in the class II MHC processing pathway (data not shown).

To demonstrate rigorously that processing at Asn 1219 was not only necessary but also sufficient for efficient presentation of TTCF, we constructed double mutants where only a single cleavage site for AEP remained. Presentation of the N1,2Q mutant of TTCF where Asn 873 and 1184 were mutated to glutamine was essentially identical to the control antigen (Figure 6). In contrast, the double mutants N1,3Q and N2,3Q behaved the same as the triple N1,2,3Q mutant. Thus, processing at site 1219 is both necessary and sufficient to initiate efficient presentation of many T cell epitopes in the TTCF antigen.

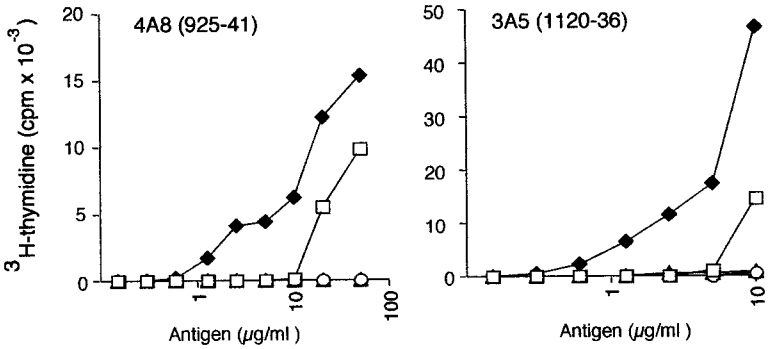

Figure 4. Presentation of the N1,2,3Q Mutant Is Not Rescued by FcγRIIB2 Receptor–Mediated Uptake

TTCF (diamonds and open squares) and the N1,2,3Q mutant (triangles and circles) were titrated with IIA1.6 B cells (H-2^d) transfected with the B2 isoform of FcγRII (Amigorena et al., 1992) with (filled symbols) and without (empty symbols) the anti-TTCF mAb 10G5 (10 μg/ml). Antigen presentation was assessed using the T cell hybridomas 3A5 and 4A8 as described in Experimental Procedures.

Discussion

Our earlier studies demonstrated that suppression of AEP activity by competitive inhibition of the enzyme in living cells slowed the presentation of a wide range of T cell epitopes in TTCF. This led us to propose a model where cleavage by AEP is the initiating and rate-limiting step for processing of TTCF (Manoury et al., 1998). However, it is difficult to absolutely exclude the possibility that the competitive peptide inhibitor used had additional effects on other processing enzymes or that AEP activity might be needed for other processing events associated with class II MHC maturation. Moreover, inhibition of AEP activity does not give information about the relative importance of the three processing sites identified. For these reasons, it was important to test our model for TTCF processing using a different strategy to inhibit AEP action on the antigen. The strict specificity of AEP for asparagine residues has allowed us to eliminate single or multiple AEP-processing sites in TTCF by mutation to glutamine.

Our data show, at least for TTCF, that processing site choice can make a crucial difference to the efficiency of antigen processing. For this antigen, efficient presentation cannot be achieved by the action of any one of several enzymes each processing multiple sites. Rather, a single enzyme acting at a single site seems to initiate a processing pathway leading to efficient presentation of many T cell epitopes. Elimination of that site does not abolish presentation, but it is dramatically slower and ~10- to 100-fold more antigen is required to elicit the same T cell response. At this point, we do not know if processing of the mutant antigen in vivo is initiated by other proteases, or alternatively if minor AEP sites are now utilized.

Consistent with the effects on T cell epitope presentation, loss of AEP-processing sites also clearly affected antigen processing at the biochemical level, as shown by an altered pattern of processed antigen fragments protected by the 11.3 antibody (Figure 2). Earlier studies showed that in vitro digestion of TTCF/11.3 antigen/antibody complexes with lysosomal hydrolases yielded a protected or "footprinted" domain of the TTCF antigen that extended from the second AEP cleavage site at Asn 1184 to the C terminus (Simitsek et al., 1995). A similar sized set of fragments is also footprinted in living cells (Davidson and Watts, 1989). It is indeed loss of Asn 1184 that is responsible for the longer fragment seen when the mutant N1,2,3Q is processed, because the same extended footprint is observed when the N2Q

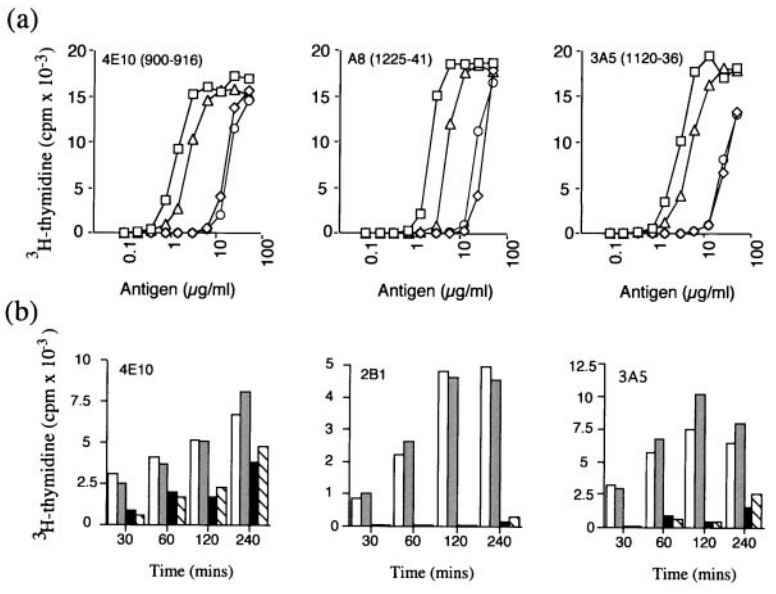

Figure 5. Cleavage at Asn 1219 Is Necessary for Efficient T Cell Epitope Presentation

TTCF (squares/white bars) N1,2,3,Q (diamonds/cross-hatching) and the single N/Q mutants N2Q (triangles/gray bars) and N3Q (circles/black bars) were tested in (a) dose-response and (b) kinetic antigen presentation assays. The assays were performed as described in Experimental Procedures and in the Figure 3 legend using LB27.4 B cells.

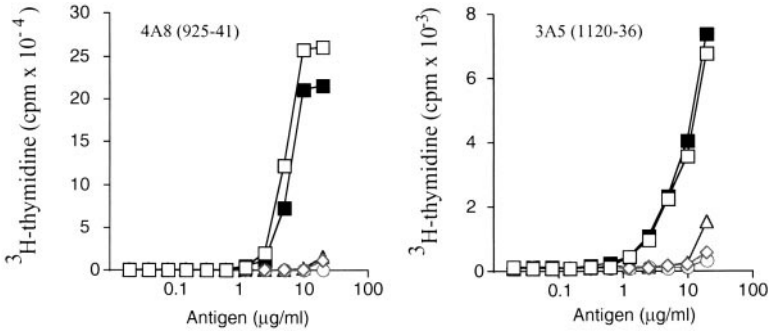

Figure 6. Processing at Asn 1219 Is Sufficient for Efficient TTCF Presentation

TTCF (open squares) N1,2,3Q (triangles) and the double mutants N1,2Q (filled squares), N1,3Q (diamonds), and N2,3Q (circles) were tested in dose-response antigen presentation assays using LB27.4 B cells and T cell hybridomas 3A5 and 4A8. The assays were performed as in Experimental Procedures and in the Figure 3 legend.

mutant was analyzed (data not shown). Interestingly, in spite of the clear effect loss of this site had on the size of the footprinted fragments, mutation of Asn 1184 to Gln (N2Q) had little or no detectable effect on presentation of the T cell epitopes that we could test.

Recently the three-dimensional structure of TTCF was solved (Umland et al., 1997; Emsley et al., 2000) and reveals a two domain structure consisting of an N-terminal "jelly roll" motif (residues 864–1107) followed by a C-terminal β-trefoil-like motif (residues 1127–1315). Asn 1219 is located on a surface loop in the latter domain (Figure 7). Cleavage at this site by AEP strongly influenced the efficiency of presentation of T cell epitopes in the C-terminal domain (e.g., those recognized by T cells 3A5 and A8). Unexpectedly, several T cell epitopes in the N-terminal domain were also markedly affected even though they are more distant in structural terms and in an apparently distinct domain. At present, we cannot fully explain the molecular basis of this striking finding. One possibility is that cleavage after Asn 1219 in living cells induces, directly or indirectly, a large-scale structural change in the protein extending to both domains. This may then facilitate further proteolysis and/or other events that ultimately lead to efficient T cell epitope loading. If this is the case, we suspect that this change may not be reproduced when crude mixtures of lysosomal hydrolases are used to digest TTCF in vitro, since under these conditions the products of AEP digestion are rather stable. Large-scale unfolding might be expected to provide a substrate for other proteases such as cathepsins D and E, which can also degrade TTCF but only after denaturation (Hewitt et al., 1997).

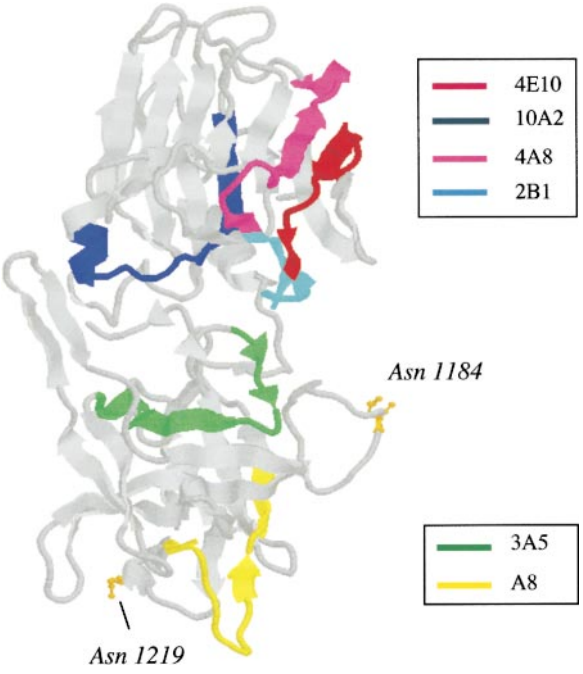

Figure 7. Long-Range Effects of Loss of a Critical Processing Site

Structure of the TTCF protein illustrating the location of the T cell epitopes used in this study and Asn 1184 and 1219. Optimal presentation of epitopes in both N- and C-terminal domains are dependent on processing at site 1219. Structure generated with RasMac using coordinates kindly supplied by P. Emsley and N. Isaacs.

Interestingly, the residue immediately downstream of Asn 1219 is also asparagine yet is clearly not a good substrate for AEP action in the N3Q mutant (Figure 1). In fact, this is one of several surface-exposed asparagine residues in TTCF that are not cleaved by AEP. However, several of these sites were shown to be efficiently cleaved by AEP when offered in the context of short (ten residues) synthetic TTCF peptide sequences, demonstrating that AEP specificity is controlled not only by Asn in the P1 position but also by the three-dimensional protein fold in which an asparagine is located (Dando et al., 1999).

Overall, our results provide evidence that there may be less redundancy in terms of protease requirement and processing site choice in a protein antigen than might have been anticipated. It should now be possible to subject other antigens and putative autoantigens to a similar analysis to that described here in order to establish the key proteases and processing sites for other protein substrates that enter the class II MHC pathway. It may well be that a requirement for specific processing proteases cutting specific processing sites is a general feature of antigen processing for presentation on class II MHC molecules. In that case, our results suggest that presentation of autoantigens and allergens may be modulated by inhibition of individual protease activities.

Experimental Procedures

Site-Directed Mutagenesis

A polyhistidine-tagged form of TTCF (Hewitt et al., 1997) was mutated in plasmid pEH101 using the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's conditions. Briefly, for each N-to-Q mutation, sense and antisense primers (see oligonucleotides) with the desired mutation were custom synthesized and

used in the following reaction protocol: 10 ng plasmid, 1 μl dNTP Stratagene mix, 2.5 U cloned pfu polymerase, 5 μl 10X reaction buffer, 125 ng sense and antisense primers, and made up to a final volume of 50 μl with distilled water and overlaid with mineral oil. The following polymerase chain reaction (PCR) was employed using a Hybaid thermocycler: 1 cycle, 95°C for 30 s; 16 cycles, 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min. Following the PCR, reactions were treated with 10 U DpnI at 37°C for 1 hr. One microliter of each reaction was then transformed into Epicurion Coli XL1-Blue supercompetent bacteria according to manufacturer's conditions. Miniprep DNA was prepared from colonies using Nucleospin Plus (Macherey-Nagel) and sequenced using the Big Dye Terminator cycle sequencing (Applied Biosystems) reaction. Positive colonies were then chosen and resequenced fully using the Big Dye chemistry and MWG Biotech sequencing services for independent verification.

Oligonucleotides

Oligonucleotides were custom synthesized by MWG Biotech. The following primers were used: for mutagenesis of Asn 873 (N1), sense primer cgt cat atg ctc gac cag gaa gac atc gat, antisense primer atc gat gtc ttc ttc ctg gtc gag cat atg acg; for Asn 1184 (N2), sense cgc tac act ccg aac cag gaa atc gat tct ttc, antisense gaa aga atc gat ttc ctg gtt cgg agt gta gcg; and for Asn 1219 (N3), sense gac ggt aac gct ttc cag aac ctg gac aga att, antisense aat tct gtc cag gtt ctg gaa agc gtt acc gtc (underlining signifies N-to-Q mutated sequence). For sequencing, the following primers were employed: vector-specific T7 and T7 terminator primers and TTCF-specific primers; sense primers caa ctt cac cgt tag ctt ctg, caa cat cac tct taa gct gga, and ttc atc aaa ctg tac gtt tct; and antisense primers tga tga tgg agt act cgt tag, ctt tgc aga aga tac gga act, and cag gtt gtt gaa agc gtt acc.

TTCF and Mutant Protein Purification

Plasmids containing the various mutations were transformed into BL21 (DE3)pLys competent bacteria. Protein was purified by growing up single bacterial colonies in 3 l LB broth/amp (100 μg/ml) and induced with IPTG (25 μg/ml) during log phase of bacterial division. Bacteria were harvested, lysed in 90 ml B-Per solution (Pierce) according to manufacturer's conditions and spun down at 27,000 × g. Supernatants were incubated with nickel agarose (Qiagen) according to manufacturers conditions, which was washed and then packed into a column. His-tagged proteins were eluted using IMAC 200 (Tris-HCl 100 mM [pH 8], NaCl 500 mM, 200 mM imidazole) and proteins were extensively dialysed against PBS.

Lysosome Preparation and TTCF Digestion

Lysosome-enriched membrane fractions were either prepared by Percoll density gradient centrifugation of postnuclear human B cell homogenates as previously described (Davidson et al., 1990) or by a combination of sucrose density gradient centrifugation and free-flow electrophoresis (Amigorena et al., 1994) from the murine B cell line IIA1.6. Wild-type and mutant TTCF substrates were digested with either purified pig kidney AEP or lysosomal protein as previously described (Manoury et al., 1998). Fragments were analyzed by SDS-PAGE on 15% Tris-tricine gels. AEP was either a kind gift from Dr. A. Barrett or was purified from pig kidney as described (Chen et al., 1997) with minor modifications.

In Vivo Processing Assay

LB27.4 cells expressing the human 11.3 BCR were incubated on ice with 2 μg/ml ^{125}I-labeled mutant or wild-type TTCF antigen at 1 × 10^7cells/ml for 1hr. Cells were then washed four times with RPMI/1% FCS wash medium. Cells were then incubated at 2 × 10^6/ml at 37°C for 0, 30, and 60 min. After each time point, cells were spun down and washed four times in wash medium, kept on ice, then lysed using 1% TX 100 immunoprecipitation lysis buffer on ice for 45 min. Lysates were then spun 14,000 rpm for 15 min and precleared with Sepharose 4B overnight. Lysates were clarified by centrifugation and the supernatants were then incubated with the anti-human κ light chain mAb QE11 (1 μl ascites; The Binding Site, Birmingham, UK). Immunoprecipitates were collected following addition of rabbit anti-mouse IgG polyclonal antisera (Serotec) and protein A Sepharose beads. Samples were boiled in Tris-tricine nonreducing loading buffer for 5 min and loaded on 15% Tris-tricine gels. Gels were dried and autoradiography performed using Konica X-ray film.

ELISA Binding Assay

Antigens were titrated across 96-well EIA flat-bottomed plates in ELISA binding buffer ($NaHCO_3$ 50 mM [pH 9.6]) and incubated overnight at 4°C. After binding, plates were washed four times in 1× PBS and then incubated with 50 μl antibody culture supernatants from B cell hybridomas 10G5, 14C8, 15D1, and 24D4 for 1h at room temperature. Plates were washed a further four times with 1× PBS, followed by incubation with 50 μl HRP-conjugated goat anti-mouse IgG (1:10,000, Pierce) for 1 hr at room temperature. The plates were developed using TMB substrate (Sigma), reactions terminated with H_2SO_4, and absorbance readings were taken at OD_{450nm}. Values are means ± SDM of triplicate points.

Generation of T Cell Hybridomas and Clones

TTCF-specific T cell clones 10A2 and A8 were generated from a C57BL/6 TTCF-specific T cell line as described previously (West et al., 1999). The 4E10 T cell hybridoma was generated from the same T cell line following fusion with the BW5147 T cell fusion partner. Briefly, a TTCF-specific T cell line and BW5147 cells were washed in serum-free medium and 1 × 10^7 of each cell type were washed together. Prewarmed PEG 1450 (0.5 ml) (Sigma) was added to the pellet over 90 s, followed by 5 ml of serum- and additive-free RPMI over 2 min and a further 15 ml over 3 min. Cells were then spun down at 1000 rpm for 5 min and gently resuspended in 50 ml cRPMI/10% heat-inactivated FCS. Cells were left at 37°C for 2–3 hr. Serial dilutions (1:4 and 1:16) were made and then plated out in 96-well flat-bottomed plates at 100 μl/well. The following day, 1× hypoxanthine selection medium was added. T cell hybridomas testing positive for TTCF specificity were maintained as described below. T cell hybridomas 2B1, 4A8, and 3A5 were obtained from a BALB/c T cell line generated in a similar manner as the C57BL/6 TTCF-specific T cell line except lymph node instead of spleen cells were used and fused with BW5147 as above. For the six T cells used in this study, the MHC restriction and peptide recognized within TTCF are as follows: 4E10 (H-2^b) TTCF 900–916, SGFNSSVITYPDAQLVP; 2B1 (H-2^d) TTCF 915–931, VPGINGKAIHLVNNESS; 4A8 (H-2^d) TTCF 925–941, LVNNESSEVIVHKAMDI; 10A2 (H-2^b) TTCF 950–966, FTVSFWLRVPKVSASHL; 3A5 (H-2^d) TTCF 1120–1136, NPLRYDTEY YLIPVASS; and A8 (H-2^b) TTCF 1225–1244, LRVGYNAPGIPLYKKME. Residue numbers are those in the complete tetanus toxin sequence (Fairweather and Lyness, 1986).

T Cell Assays

Kinetic Assays

LB27.4 cells were incubated at 5 × 10^6 cells/ml with 300 μg/ml of antigen at 37°C. The cells were then harvested, washed three times, fixed for 4 min in 0.5 ml paraformaldehyde (0.5%), and after further washing, cultured at 1 × 10^5/well with T cells. T cell hybridomas were harvested and used at 1 × 10^5/well and T cell clones were used at 1 × 10^4/ well. After 24 hr, assays were frozen and supernatants tested subsequently for IL-2 using HT-2 indicator cells (see below).

Antigen Titration Assays

Antigen was titrated using 2-fold dilutions across 96-well U-bottomed plates in HL-1 medium (Biowhittaker). APCs and T cell hybridomas were added at 1 × 10^5/well, while T cell clones were added at 1 × 10^4/well. For FcR/antibody-mediated antigen presentation assays, antigen was titrated as above and 10G5 TTCF-specific antibody was added at 10 μg/ml and incubated at 4°C for 1hr. IIA1.6 FcγRIIB2 transfectants (1 × 10^5) were added per well along with 1 × 10^5 T cell hybridomas. Plates were frozen after 24 hr and the supernatants were tested for IL-2 using HT-2 cells.

HT-2 Proliferation Assays

HT-2 cells were incubated at 5 × 10^3/well with 50 μl supernatants. After 24 hr, wells were pulsed with 1 μCi [^{3}H]thymidine (Amersham). Sixteen to eighteen hours later, the plates were harvested onto nitrocellulose filters (Tomtek) and ^{3}H incorporation was measured using the 1450 Microbeta Plus liquid scintillation counter. ^{3}H incorporation was expressed as the mean cpm of duplicate points.

Cell Lines and Media

LB27.4 (H-2^{bxd}) a kind gift from A. Livingstone, was maintained in RPMI supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml kanamycin, and 50 μM 2-Me (cRPMI). LB27.4 11.3 BCR transfectants were maintained in cRPMI/10% FCS and G418 (0.4 mg/ml) and hygromycin (0.3 mg/ml) drug selection. HT-2 cells (a kind gift from Paul Fairchild) were maintained in cRPMI and 0.5% IL-2 supernatant at 1×10^4 cells/ml. T cell hybridomas 4E10, 2B1, 4A8, and 3A5 were maintained at 5×10^3/ml in RPMI-25mM HEPES supplemented with 10% heat-inactivated FCS, 2 mM glutamine,100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-Me. T cell clones were maintained at 1×10^4 cells/well with irradiated 5×10^6 C57BL/6 spleen cells/well, TTCF (0.5 μg/ml) and 2%–3% rat T cell growth factor. BW5147 T cell fusion partner (a kind gift from Dr. P. Chandler) was maintained in cRPMI/10%FCS. IIA1.6 FcγRIIB2 (kindly provided by S. Amigorena and C. Bonnerot) transfectants were maintained in cRPMI with 1 mg/ml G418 selection.

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